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Biochemistry of Protein-Isocyanate Interactions: A Comparison of the Effects of Aryl vs. Alkyl Isocyanates

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In addition to their use in the polyurethane and pesticide industries, isocyanates have proven to be useful probes for the exploration of protein structure. This paper focuses on three aspects of isocyanates: their broad reactivity, their reversible interaction with cholinesterases, and the relative hydrolysis rates of alkyl and aryl isocyanates. The broad reactivity of isocyanates as well as the demonstrated affinity labeling of serine and sulfhydryl esterases are discussed. Extension of the affinity labeling studies to include the analysis of the inhibition of cholinesterases by methyl isocyanate shows that methyl isocyanate is not an effective inhibitor of any of the cholinesterases. The inhibition of cholinesterases by alkyl isocyanates shows a pattern of decreased specificity with decreased alkyl chain length. The inhibition of cholinesterases by isocyanates is shown to be reversible, with a maximum rate of reversal seen at physiological pH. This reversal is characteristic of the reaction of an isocyanate with a sulfhydryl group. Finally, the affinity labeling of proteins must compete successfully with the hydrolysis of isocyanates in aqueous solution. The hydrolysis of alkyl isocyanates is shown to be significantly slower than that of the aryl isocyanates.

The application of alkyl and aryl isocyanates to the pesticide and polyurethane industries is of major commercial interest worldwide. In addition, the isocyanates have proven to be valuable tools to protein chemists making correlations between protein structure and function. It is the information from these latter studies that may prove valuable to our understanding of how isocyanates affect human and animal populations. As a reagent used for the study of protein structure and function, isocyanates have two major characteristics that make them attractive. First, they are highly reactive with a variety of functional groups found on biological macromolecules, and second, they have a finite lifetime that enables them to react with selected functional groups but not to exist long enough to cause significant, nonspecific modification of the macromolecule under study. It is the purpose of this paper to explore both of these advantages of the isocyanates as protein modification reagents, to summarize information already in the literature, and to present new data relevant to each point. Of particular interest to this symposium will be the comparison of the biochemical results for the aryl isocyanates and the alkyl isocyanates. In the former case, the emphasis will be on toluene diisocyanate (TDI) for which there is extensive toxicological information (*1*), and in the latter case, the emphasis will be on methyl isocyanate (MIC), which is the focus of this symposium.

The first point of discussion will be the reactivity of the isocyanates. Table 1 shows the typical reactions of isocyanates with different functional groups that occur on proteins and other biological macromolecules. Virtually every functional group gives a defined product with an isocyanate under some defined set of conditions. For the purpose of this paper the discussion will center only on those products that readily form under physiological conditions and thus are potential sites of reaction under exposure conditions. This narrows the candidates to the hydroxyl group (reaction 2), the sulfhydryl group (reaction 3), and the imidazole group (reaction 5). Although the other reactions are possible, they are less probable because significantly reduced levels of the appropriate reactive form of the functional group exist under physiological conditions. For example, the amine reaction with the isocyanate is optimal with a nonprotonated amine, and under physiological conditions less than 5% of the protein amines are nonprotonated. As will be discussed later, the reaction of the isocyanates with the hydroxyl group of serine has been demonstrated to be the result of the modification of the serine esterases (*7*). The modification of the tyrosyl-hydroxyl has been demonstrated with model compounds (*8*) but has not been observed in proteins. In

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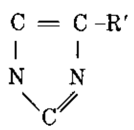
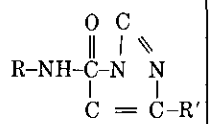
Table 1. Reactions of isocyanates (R–N=C=O) with biologically relevant functional groups.

Reactive functional group	Product	Reference
$H_2N{-}R'$	$R{-}NH{-}C(=O){-}NH{-}R'$	(*2*)
$HO{-}R'$	$R{-}NH{-}C(=O){-}O{-}R'$	(*3*)
$HS{-}R'$	$R{-}NH{-}C(=O){-}S{-}R'$	(*4*)
$HO{-}C(=O){-}R'$	$R{-}NH{-}C(=O){-}O{-}C(=O){-}R'$	(*5*)
	$R{-}NH{-}C(=O){-}R' + CO_2$	
		(*6*)
H_2O	$R{-}NH_2 + CO_2$	

addition, the reaction with hydroxyl, R′OH, shown in Table 1 is analogous to the hydrolysis reaction of the isocyanate that results in the formation of carbon dioxide and the corresponding amine (Table 1). It is important to note that any site-specific reaction that one describes is occurring in successful competition with this hydrolysis reaction. This is an important consideration in the case of the imidazole nitrogen reaction since although it is a good nucleophile at physiological pH, the reaction of secondary amines is slow relative to the rate of isocyanate hydrolysis.

Of particular interest is the reaction with the sulfhydryl group that can occur at physiological pH, whose product is stable under acidic conditions, but which is fully reversible under slightly alkaline pH conditions (*8*). It is worth noting that the reversal of this reaction proceeds through formation of the isocyanate (*4*). The implication of this observation will be discussed later. Finally, the imidazole group is a potential site of modification at physiological pH, and, although model compounds have been synthesized, this specific modification has not been observed in proteins to date. All of the aforementioned reactions take place with both the alkyl and aryl isocyantes.

The diverse reactivity of the isocyanates make them attractive as potential molecular "rulers" to measure structural features of proteins. To this end, studies were performed to measure the intramolecular distance between amino acids on a protein by crosslinking amino acid side chains with bifunctional isocyanates of defined dimensions (*9*). This analysis was extended to monofunctional isocyanates in the case of the serine proteases (chymotrypsin, trypsin, and elastase), and it was found that not only did the isocyanates discriminate between different esterases but they also demonstrated a chain-length dependence for inhibition of a single esterase (*7*). Subsequent biochemical analysis showed that the reaction was irreversible even in the presence of strong nucleophilic reagents, the modification was on the active-site serine 195 in the case of chymotrypsin (*10*), and that the alkyl chain was buried in the hydrophobic substrate-binding pocket of the enzyme (*11*). Thus the alkyl isocyanates had been shown to be excellent active-site directed affinity probes for the serine esterases. It is worth noting that although these were *in vitro* experiments, they were carried out at pH 7.5 and represent stoichiometric inhibition of the enzymes in aqueous medium.

Subsequent to this work, *in vitro* titrations of other enzymes were performed; noteworthy among these were papain and alcohol dehydrogenase (*8*), which were both inhibited by butyl isocyanate. The major differences between these studies and those previously cited for the serine esterases are that the inhibition of both of these enzymes results from modification of their active site sulfhydryl group and that the inhibition by the isocyanates was reversible under slightly alkaline pH (pH $\geqslant$ 7.0).

Finally, it has been shown that in *in vitro* titrations of the cholinesterases with a variety of isocyanates, both alkyl and aryl, there is both inhibitory discrimination of a single isocyanate for different cholinesterases and of different isocyanates for a single cholinesterase (*12*). This effect is similar to the same effect seen with other anticholinesterases such as the organophosphates and carbamates and has been discussed elsewhere (*13*). The purpose of this paper is to extend the characterization of the cholinesterase-isocyanate interaction to include methyl isocyanate and to demonstrate that MIC is not an effective inhibitor of the cholinesterases. Furthermore, it will be shown that the inhibition of the cholinesterases by other isocyanates is fully reversible under physiological conditions.

The second point to be made in this paper is that these site-specific stoichiometric inhibitions of esterases by isocyanates and indeed any site-directed affinity labeling of a protein by any isocyanate must compete successfully with the simultaneous hydrolysis of the isocyanates by water. Though substantial information is available on the respiratory effects of the aryl isocyanates (*1*) and much has been presented at this meeting on the effects of methyl isocyanate, little information has been presented on the lifetime of the different isocyanates in aqueous media. The results presented in this paper will show that the alkyl isocyanates are significantly more stable than the aryl isocyanates against hydrolysis. This extended lifetime of MIC may account for the significant differences seen in the inhalation irritation between TDI and MIC.

Methods

Materials

Horse serum cholinesterase (12.9 units/mg) and eel cholinesterase (355 units/mg) were purchased from Sigma Chemical Company and used without further purification. Human serum cholinesterase was purified from plasma obtained from the Central Blood Bank of Pittsburgh, PA. Purification to a specific activity of 150 units/mg was achieved by a combined purification scheme of Main et al. (*14*) and Lockridge et al. (*15*). Hexyl, butyl, *o*-tolyl, and *p*-tolyl monoisocyanates were purchased from Eastman Organic Chemicals and have a purity of 98% or greater. Methyl isocyanate and hexamethylene diisocyanate were purchased from Aldrich Chemical Company.

Enzyme Assays

Horse and human serum cholinesterases were assayed according to the procedure of Ellman et al. (*16*) with butyrylthiocholine (Sigma) as substrate. Eel cholinesterase was assayed by the same procedure, acetylthiocholine (Sigma) being used as substrate. One unit of activity was defined as the hydrolysis of 1 μmole of butyrylthiocholine (or acetylthiocholine) per minute at 25°C.

Cholinesterase Titration with Isocyanates

The titration of the cholinesterases was carried out exactly as described previously (*12*). Initial protein concentrations were 1 mg/mL in 20 mM phosphate buffer, pH 6.9, and the isocyanates were added as aliquots from stock solutions of isocyanates prepared in acetone. The isocyanates were dissolved in acetone to facilitate their solubility and dissolution in the aqueous reaction mixture. The acetone never exceeded 10% of the final reaction volume and was found to have no effect on cholinesterase activity in parallel experiments. The results are expressed as percent of the initial activity of cholinesterase.

Reversal of Isocyanate Inactivation

Human serum cholinesterase (1 mg/mL) was titrated with hexamethylene diisocyanate (HDI) as described in the preceding section to 28% of its initial activity. The final reaction mixture was adjusted to a pH of 7.5 and incubated at room temperature. Aliquots of the adjusted reaction mixture were removed over a 26-hr time period and immediately assayed for cholinesterase activity. The results are expressed as the percent of the sample's pretitration activity as a function of time. Samples titrated to lower initial remaining activity showed recovery to a lower final level (data not shown).

pH Dependence of Reversibility

Human serum cholinesterase was titrated to 28% of its initial activity with HDI at pH 6.9 as previously described. Aliquots of the final reaction mixture were incubated at different pH values following titration with either acid (HCl) or base (NaOH) to the desired pH. Aliquots from each of these incubations were removed at prescribed time intervals, diluted into the assay buffer, and immediately assayed in the standard assay protocol. This procedure generated curves similar to that in Figure 2 for each pH under study. From each curve, the initial velocity for the recovery of activity at a defined pH is measured. The final results are then expressed as the rate of reactivation as a function of pH.

Measurement of Hydrolysis Rates

The rates of hydrolysis of hexamethylene monoisocyanate (HMI) and of *o*- and *p*-toluene monoisocyanate (TMI) are being determined using proton nuclear magnetic resonance (NMR). All results reported here were performed on the Bruker WH300 NMR instrument in Dr. Chien Ho's laboratory at Carnegie-Mellon University. Utilization of NMR for the kinetic studies made it possible to accurately measure the quantity of water present in the hydrolysis experiment, the quantity of isocyanate present, the rate of disappearance of the isocyanate, and the type of and rate of appearance of all products and intermediates. This paper reports only the rate of isocyanate disappearance while a future publication will provide a complete analysis of the hydrolysis of these monoisocyanates and certain diisocyanates. All hydrolysis experiments were performed under similar conditions, and thus a typical protocol will be given. Experiments were performed in both deuterated dimethyl sulfoxide (DMSO) and deuterated acetone. These solvents were chosen because the solubility of both water and the isocyanates in them provide a uniform miscible system. Acetone was the preferred solvent in later experiments due to the observed catalytic effect DMSO had on the hydrolysis reaction. A NMR tube was filled with 1.0 mL of solvent, and the spectrum was measured to determine the amount of water present in the sample. A measured quantity of water was then added to the solvent such that the concentration was between 0.11 and 3.33 M for these experiments. A spectrum was taken to verify the water concentration. At zero time, a measured quantity of isocyanate was added to the water-solvent mixture, and spectra were taken at defined intervals. The first data point was collected within 1.5 min of introduction and mixing of the isocyanate, and data collection was computer-controlled, with the most rapid sequence of time points being 30-sec intervals. For each isocyanate, defined resonances were identified that represented the unhydrolyzed isocyanate—for example, the protons on the alpha carbon for HMI. The second-order rate constants were derived

from the slopes of the linear second-order plots for the disappearance of the isocyanate in each case. The reported rate constants represent the average of at least three experiments performed at concentrations of isocyanate ranging from 0.019 to 1.08 M and concentrations of water ranging from 0.11 to 3.33 M with ratios of water to isocyanate ranging from 0.4 to 10.

Results and Discussion

In Vitro Effect of MIC on Cholinesterases

In experiments performed under conditions similar to those previously used (*12*), eel acetyl cholinesterase and equine serum butyryl cholinesterase were titrated with methyl isocyanate. The results of these titrations are illustrated in Figure 1. It can be seen that although there is some discrimination between the two cholinesterases for methyl isocyanate, neither of the enzymes are effectively inhibited by MIC. For comparison the titration of serum cholinesterase by HDI is shown on the graph to illustrate the effect of a stoichiometric inhibitor. Compiled in Table 2 is a comparison of the effect of different alkyl and aryl mono- and diisocyanates on the two cholinesterases. The diisocyanates generally appear to be the more potent inhibitors of the cholinesterases, as discussed previously (*12*), while the alkyl monoisocyanates show a marked dependence on chain length, with the shorter chain isocyanates being the poorer inhibitors. This is similar to the effect observed with the inhibition of chymotrypsin by the alkyl isocyanates and may reflect a decrease in the specific affinity making the protein reaction less competitive with the hydrolysis reaction.

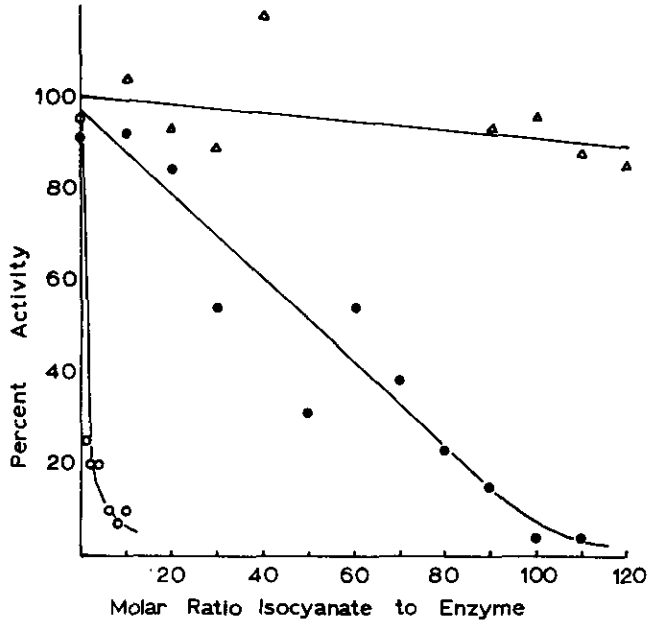


FIGURE 1. Titration of cholinesterases with methyl isocyanate: horse serum cholinesterase in 20 mM phosphate buffer titrated with (●) methyl isocyanate and (○) hexamethylene diisocyanate; (△) eel acetyl cholinesterase, under the same conditions titrated with methyl isocyanate.

Table 2. Inhibition of cholinesterases by isocyanates at various molar ratios of isocyanate/protein for 50% enzyme inhibition.

Isocyanate	Isocyanate/protein ratio for 50% inhibition[a]	
	Horse serum enzyme	Eel enzyme
Methyl isocyanate	51	560
Butyl isocyanate	10	109
Hexyl isocyanate	4.0	55
Hexamethylene diisocyanate[b]	3.2	1.8
2,6-Toluene diisocyanate[b]	0.8	202
2,4-Toluene diisocyanate[b]	68	700

[a] Calculations of molar ratios assumed that all protein was cholinesterase.

[b] Data taken from a previous study (*12*) for comparison. Experiments were performed under identical conditions.

Reversible Nature of Isocyanate Inhibition of Cholinesterases

Even though MIC is not an effective inhibitor of cholinesterase, it is relevant to examine the characteristics of the isocyanate-cholinesterase conjugates formed by other isocyanates. In light of the reversible nature of the BIC-papain and BIC-alcohol dehydrogenase modifications (*8*), studies were conducted on the reversibility of the isocyanate-cholinesterase conjugates. In findings reported earlier (*17,18*), it was found that upon incubation of isocyanate-cholinesterase conjugates at pH 7.5, there is almost complete recovery of the enzymatic activity. Reported herein are the previously unpublished details of the experiments performed by our group. Figure 2 shows the rate of the recovery of enzymatic activity at pH 7.5 for a sample of human serum cholinesterase which had been initially inhibited by HDI to 28% of control activity. Two characteristics of the recovery can be seen in this plot. The first is the time for reversal, this process appears to be complete within 7 hr. The second characteristic is that the reversal is essentially complete (89% of initial activity). This latter observation should be qualified by the fact that the cholinesterase was not completely inhibited (i.e., the initial reaction product of the isocyanate with the cholinesterase retained 28% of its pre-inhibition activity). This initial value for the reversibility experiments was chosen because, as seen previously (*12*), the inhibition titration curve is linear to this point, indicating a minimum of nonspecific modification of the enzyme. Beyond this point, nonspecific inhibition begins to appear that may result in loss of activity by second site modification. Loss of complete reversibility below this point may reflect either involvement of a second irreversible modification that may also result in enzyme inhibition or irreversible denaturation of the enzyme as a result of the more extensive modification.

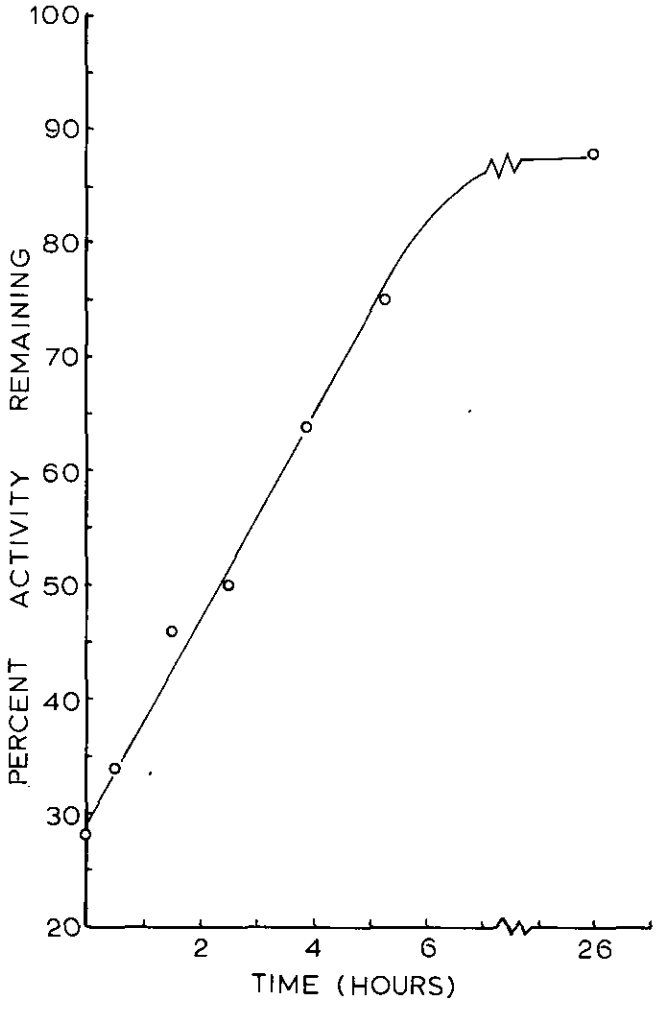


FIGURE 2. Rate of recovery of cholinesterase activity following inhibition by HDI. Human serum cholinesterase, inhibited to 28% of its original activity, is incubated at room temperature in 20 mM phosphate at pH 7.5.

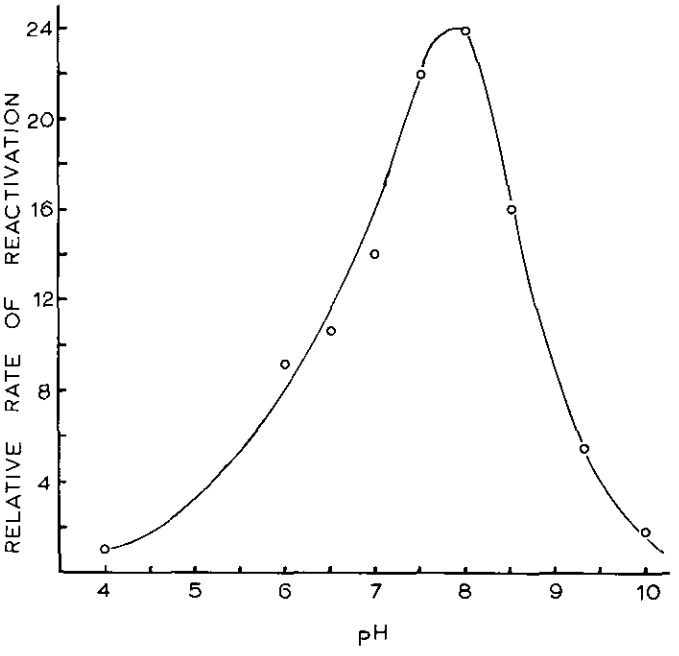


FIGURE 3. pH dependence of the recovery of cholinesterase activity following inhibition by isocyanate. Human serum cholinesterase, inhibited to 28% of its original activity by HDI, is incubated at room temperature under a variety of pH conditions. The initial rate of reactivation at each pH was measured from plots similar to those in Fig. 2.

pH Dependence of the Reversibility

The reversibility shows a marked pH dependence as illustrated in Figure 3. The low pH stability and alkaline pH reversal are similar to those seen with the modification of the active site sulfhydryl in papain and alcohol dehydrogenase (*3*). Unlike papain and alcohol dehydrogenase, however, this loss of reactivation at high pH is not due to irreversible denaturation of the enzyme at these pH values since control experiments show retention of activity of unmodified enzyme up to pH 10. This loss of reactivation may be due either to induced instability in the enzyme as a result of the initial modification by the isocyanate or to shift of the isocyanate from a reversibly modified active site amino acid (i.e., a sulfhydryl group) to an irreversibly modified active-site amino acid (e.g., a hydroxyl or an amino group). As mentioned above, the modification of a sulfhydryl by an isocyanate yields a product that is reversible under slightly alkaline pH and proceeds through the isocyanate functional group before either being hydrolyzed or reacting with another strong nucleophile, such as the amino group above its pK_a. This suggests that a sulfhydryl containing protein or peptide may act as a carrier of the isocyanate group for possible reaction upon its release at a later time and/or place. Although no direct evidence exists for this mechanism under physiological conditions, it is certainly a hypothesis that can be tested with the use of labeled isocyanates.

Relative Hydrolysis of Alkyl and Aryl Isocyanates

Because of the vast amount of information available on the respiratory effects of TDI (*1*) and the most recent observations of the respiratory effects of MIC (*19*), it was thought that the finding of pulmonary sensitization observed with MIC and not with TDI under similar conditions might be explained by the difference in their lifetime in aqueous environments. From previously published data (*7*) it was found that the rate of disappearance of the alkyl isocyanates, BIC and HMI, was second-order as expected and was the same for the two alkyl isocyanates within experimental error. This would suggest that there may be no significant difference in the rate of hydrolysis of the alkyl monoisocyanates as a function of the alkyl chain length. Thus MIC would have a half-life of approximately 2 min in an aqueous phosphate-buffered solution at pH 7.5 (the conditions chosen for the *in vitro* inhibition studies of the serine

esterases). As mentioned previously, the finite lifetime of a reagent is a characteristic that is beneficial in *in vitro* protein chemical modification studies since the reagent would be stable enough for site-specific modifications but would hydrolyze before extensive nonspecific modifications would take place. However, under *in vivo* physiological conditions, where a variety of different proteins exist simultaneously at very high concentrations, the potential for nonspecific modification is greatly increased. Therefore, the longer a reagent's reactive lifetime, the more potential a reagent has for extensive modification of biological macromolecules. To understand the differences in the pulmonary effects of MIC and TDI, it thus became important to determine the relative difference in hydrolysis rates between the alkyl and aryl isocyanates. Table 3 lists the second-order rate constants determined for the hydrolysis of HMI and *o*- and *p*-toluene monoisocyanates (*o*-TMI, *p*-TMI). Although performed at stoichiometric rather than saturating conditions of water concentration, the relative hydrolysis rates of the alkyl and aryl isocyanates would indicate a 5- to 1400-fold faster rate for the hydrolysis of the aryl monoisocyanates over the alkyl monoisocyanate, depending on the isomer of aryl isocyanate and the solvent chosen for the study. It is known that DMSO enhances certain organic reactions and thus the data collected in acetone may be the most significant; however, emphasis should be placed on the relative comparison of the rates in each solvent rather than between solvents. These results would support the speculation that the greater pulmonary sensitization seen with the MIC relative to TDI (*19*) may in part be due to the longer lifetime of MIC in the airways due to a relatively slower rate of hydroysis. These rates are meant to illustrate relative rates and at this point in our investigation do not represent absolute values for the aqueous hydrolysis rates.

In summary, the data thus far would indicate that MIC has a relatively long lifetime, is highly reactive, and is not an anticholinesterase reagent. In addition, the lack of efficient inhibition of cholinesterase by MIC and the reversibility of the inhibition by other isocyanates in the *in vitro* experiments might explain the inability of *in vivo* exposure experiments to show any effect on serum cholinesterase (*20*). Thus the destructive nature of MIC may be due to indiscriminant labeling of biological macromolecules. At this point it is worth mentioning the recent presentation of data from the exposure of guinea pigs to radioactively labeled TDI (*21*). The results of these studies indicate that TDI is taken up in the blood of the animals in a dose-response manner with no significant difference between animals previously sensitized to TDI and nonsensitized animals. In addition, all of the radioactivity in the blood of the exposed animals appears irreversibly attached to a single protein species of 71,000 molecular weight. These results would indicate a very specific mechanism for the interaction of TDI with the physiological system. Although the data reported here would indicate real differences between the alkyl and aryl isocyanates in their reaction specificity and hydrolysis rates, it is intriguing to speculate as to whether there is a similarity in the uptake of the MIC and TDI physiologically. Again, this is a question that can be resolved with the aid of a labeled MIC probe.

Table 3. Second-order rate constants for the hydrolysis of isocyanates under controlled aqueous conditions.

Isocyanate	Second-order rate constant/M-min	
	In DMSO	In acetone
Hexamethylene monoisocyanate	5.4×10^{-3}	4.1×10^{-4}
o-Tolyl monoisocyanate	1.8×10^{-1}	1.9×10^{-3}
p-Tolyl monoisocyanate	7.68	ND

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